Analysis of Interactions of EF-G and EF-Tu with the Ribosome

Honors Research Thesis

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by
Emily DeDonato

The Ohio State University
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Project Advisor: Dr. Kurt Fredrick, Department of Microbiology

ABSTRACT:

Guanosine triphosphate hydrolases (GTPases) play important roles in many cellular processes, including communication, division, and transport. Two specific GTPases, Elongation Factor Tu (EF-Tu) and Elongation Factor G (EF-G), catalyze different steps of the elongation phase of translation. Although these are extensively studied GTPases, how EF-Tu and EF-G interact with the ribosome is still not fully understood. In order to investigate the interactions between these elongation factors and the ribosome, I analyzed how mutations in helix 5 (h5) of the 16S rRNA of *Escherichia coli* affect translocation (EF-G function) and decoding (EF-Tu function). Single-point mutations were made at positions 55, 357, and 367 of 16S rRNA and the corresponding ribosomes were purified using affinity chromatography. To determine the effects of the mutations on translocation, the rate of tRNA-mRNA movement was measured under single-turnover conditions. Mutations in h5 had minimal effects on translocation, suggesting that these nucleotides are not critical for the binding or function of EF-G. To determine the effects of the mutations on decoding, apparent rates of EF-Tu-dependent GTP hydrolysis were determined. Mutations A55C and U367G were found to dramatically reduce the rate of GTP hydrolysis, suggesting a specific defect in EF-Tu function. This work sheds light on the role of specific ribosomal residues in elongation. This information will add to our knowledge of translation at the molecular level, and may lead to useful tools for protein engineering.

INTRODUCTION:

Roles of EF-G and EF-Tu in Elongation

Translation is the synthesis of proteins by ribosomes using messenger RNAs (mRNA) as templates and aminoacyl-transfer RNAs (aa-tRNA) as substrates. The process of translation occurs in three phases: initiation, elongation and termination. The elongation phase consists of the steps involved in adding amino acids to the peptide chain of the protein. An important family of GTPases are involved in elongation. The related GTPases EF-G and EF-Tu are responsible for catalyzing different steps of the elongation cycle. EF-Tu brings aminoacyl-tRNA to the

ribosome A site and helps to select for the correct codon-anticodon pairing (decoding). EF-G is responsible for promoting translocation of tRNA (paired to mRNA) in the ribosome after peptide bond formation. Although they perform different functions during translation, these two GTPases interact with the ribosome in a similar fashion. Domains I and II of the factors are homologous and domain II interacts with helix 5 (h5) of the 16S ribosomal RNA (rRNA), located on the shoulder of the 30S subunit of the ribosome.

Background

In a previous study, the effects of mutations within h5 of the 16S rRNA (positions 55, 357, and 367) were shown to lower translational activity in the cell dramatically[3]. These single base substitutions were generated at positions 55, 357, 367. The molecular mechanism behind how these mutations influence translation remains unknown. Clues about how h5 interacts with EF-Tu and EF-G come from crystal structures of the factors bound to the ribosome (Figure 1). By comparing the crystal structure of the ribosome bound to the EF-Tu ternary complex (EF-Tu·GTP·aminoacyl-tRNA) with the crystal structure of the ribosome bound to EF-G, it is apparent that the two GTPases, while similar in their binding locations, do display some differences in their interaction with 16S rRNA. For example, EF-Tu·GTP·aminoacyl-tRNA and EF-G interact differently with the A55 residue of the 16S rRNA. In the EF-Tu-bound structure, the A55 base moves out from h5 to stack with C75 of the incoming A-site tRNA. In the EF-G-bound structure, A55 is tucked within h5 and appears to form no base-specific contacts. Another difference exists in the interaction between the ribosome and domain III of the two factors. Domain III of EF-G interacts with h5, while domain III of EF-Tu does not appear to be anywhere near h5 in the protein-ribosome complex[1,5].

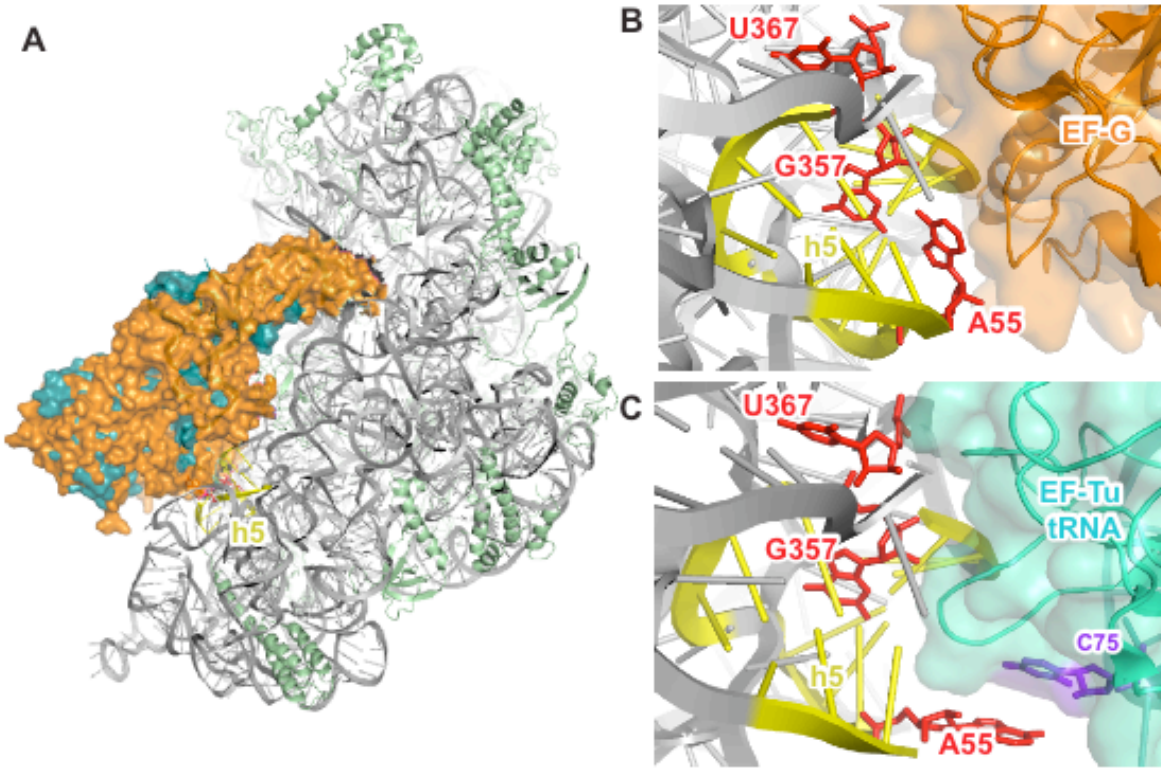

Figure 1. Crystal Structure of ribosome-factor complexes[1,5]. (A) An overview of the binding site of EF-G and EF-Tu on the 30S subunit. Color scheme: 16S rRNA, gray; 30S proteins, green; EF-G, orange; EF-Tu·tRNA, blue; h5 yellow. (B-C) Detailed interaction of EF-G (B) and EF-Tu·aa-tRNA·GDP (C) with h5 of the 16S rRNA, key residues from h5 are highlighted in red and C75 of the tRNA is highlighted in purple.

METHODOLOGY:

Point Mutations on 16S rRNA

Single-point mutations were made at positions 55, 357, and 367 on the 16S rRNA gene of plasmid pEY35spurMS2 through quick-change reactions. This plasmid has an MS2 aptamer engineered into the spur region of the 16S rRNA gene, which allows for purification of the encoded mutant ribosomes using affinity chromatography[9]. Primers used were as follows:

A55C
Forward: 5’ CAGGCCTAACAC**C**TGCAAGTCGAACG 3’
Reverse: 5’ CGTTCGACTTGCA**G**GTGTTAGGCCTG 3’

A55G
Forward: 5’ CAGGCCTAACAC**G**TGCAAGTCGAACG 3’
Reverse: 5’ CGTTCGACTTGCA**C**GTGTTAGGCCTG 3’

A55U
Forward: 5’ CAGGCCTAACAC**U**TGCAAGTCGAACG 3’
Reverse: 5’ CGTTCGACTTGCA**A**GTGTTAGGCCTG 3’

G357U
Forward: 5’ GGGAGGCAGCA**U**TGGGGAATATTGC 3’
Reverse: 5’ GCAATATTCCCCA**A**TGCTGCCTCCC 3’

G357A
Forward: 5’ GGGAGGCAGCA**A**TGGGGAATATTGC 3’
Reverse: 5’ GCAATATTCCCCA**U**TGCTGCCTCCC 3’

G357C
Forward: 5’ GGGAGGCAGCA**C**TGGGGAATATTGC 3’
Reverse: 5’ GCAATATTCCCCA**G**TGCTGCCTCCC 3’

U367G
Forward: 5’ CAGTGGGGAATA**G**TGCACAATGGGCG 3’
Reverse: 5’ CGCCCATTGTGC**C**ATATTCCCCACTG 3’

U367A
Forward: 5’ CAGTGGGGAATA**A**TGCACAATGGGCG 3’
Reverse: 5’ CGCCCATTGTGC**U**ATATTCCCCACTG 3’

U367C
Forward: 5’ CAGTGGGGAATA**C**TGCACAATGGGCG 3’
Reverse: 5’ CGCCCATTGTGC**G**ATATTCCCCACTG 3’

Purify Ribosomes using Affinity Chromatography[9]:

Ribosomes that carried the MS2 aptamer tag were expressed in DH10 cells that carry pcI857 (Kan^R) which encodes a temperature sensitive lambda repressor protein that represses rRNA transcription from pEY35spurMS2 at 30°C. The cell cultures were grown overnight at 30°C in LB with 100 ug/mL of ampicillin and 30 ug/mL of kanamycin. Then the cell cultures were grown to mid-log phase (OD600 0.5-0.6) in 1 L of LB with 100 ug/mL of ampicillin. Next, the expression of the tagged ribosomes was induced by growing the cultures in LB at 42°C for two hours. The cultures were then chilled on ice (0.5 hr) and pelleted. The pellets were then resuspended in 30 mL of ribosome buffer A (20 mM Tris-HCl pH 7.6, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 M EDTA and 6 mM BME) and lysed with a French Press. The lysate was then spun at 15,000 rpm using a JA20 rotor for 15 minutes. The supernatant was layered onto 10 mL

sucrose buffer cushions (37.7% sucrose, 20 mM Tris-HCl pH 7.6, 500 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 M EDTA and 6 mM BME) in Ti 60 tubes. The tubes were filled to shoulder with ribosome buffer B (20 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 500 mM NH_4Cl, 0.5 EDTA and 6 mM BME). The ribosomes were pelleted by ultracentrifugation at 33,200 rpm using a Ti 50.2 rotor (Beckman) for 21 hr at 4°C. The pellets were rinsed with ribosome buffer A then dissolved in ~1 ml of the same buffer. The ribosome solution was then added to Ti 60 tubes and filled with ribosome buffer B. The ribosomes were pelleted by ultracentrifugation at 36,300 rpm using a Ti 50.2 rotor for 2.5 hr. Pellets were then dissolved in binding buffer (20 mM Tris-HCl pH 7.6, 1 mM $MgCl_2$, 100 mM NH_4Cl, 6 mM BME) and dialyzed against binding buffer overnight.

A pre-equilibrated 5 mL GSTrap FF column was loaded with GST-MS2 fusion protein (6mg) with a flow rate of 0.5 mL/min, and then the column was washed with 15 mL of binding buffer. Next, between 90-150 mg of crude ribosome was loaded onto the column at 0.5 mL/min and then the column was washed with 25 mL of binding buffer. Tagged ribosomes bound to the GST-MS2 protein were then eluted with 20 mL of elution buffer (50 mM Tris-HCl pH7.6, 1 mM $MgCl_2$, 100 mM NH_4Cl, 10 mM reduced glutathione, and 6 mM BME). Fractions with A260 readings of >0.1 were combined and concentrated using Amicon Ultra-4 (100,000 MWCO). They were then dialyzed against storage buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM NH_4Cl and 6 mM BME) overnight. Lastly, they were flash frozen and stored at -80°C.

Determine Effects of the Mutations on EF-G-dependent Translocation

The apparent rates of EF-G catalyzed translocation were measured under single-turnover conditions for mutant and wild-type ribosomes. 70S ribosomes were formed by heat activating the 30S subunits at 42°C for 20 min and then adding the 50S subunit and incubating at 37°C for 15 min. Message m625 (5’ -AAGGAAAUAAAAAUGGUAUAU -3’) with a 2’amino-pyrene modification at the 3’-end was used so that the tRNA-mRNA movement could be monitored by fluorescence stopped-flow[6]. As the tRNA-mRNA moves in the 5’-direction (relative to the ribosome), the fluorescence is quenched. The fluorescence decrease is then monitored as a function of time and the data are fit to an exponential curve. The P-site complex was formed by incubating the 70S ribosomes (1.5

μM) with $tRNA^{Met}$ (1.5 μM) and m625 (1.25 μM) in buffer (50 mM Tris-HCl pH 7.6, 100 mM NH_4Cl, 15 mM $MgCl_2$ and 6 mM BME) at 37 °C for 20 min. Next, N-acetyl-Val-$tRNA^{Val}$ (1.5 μM) was added to bind to the ribosome A site and incubated at 37°C for 20 min. The pre-translocation ribosome complex was diluted 5-fold before mixing with EF-G·GTP in the SX20 stopped-flow machine (Applied Photophysics). The concentration of EF-G used was varied throughout the experiment in order to determine the kinetic parameters, k_{cat} and K_M of the reaction.

Determine the Effects of the Mutations on EF-Tu-dependent GTP hydrolysis

In order to determine the effects of the mutations on decoding, I measured the rate of EF-Tu catalyzed GTP hydrolysis in single-turnover conditions with excess ribosomes. Ternary complex was formed by mixing α-[^{32}P]-GTP with EF-Tu (12.25 μM), pyruvate kinase, PEP, and Phe-$tRNA^{Phe}$ in buffer A (25 mM Tris HCl pH 7.5, 50 mM NH_4Cl, 1 mM DTT, 5 mM $MgCl_2$) and incubating at 37°C for 5 min. 60 pmol of ternary complex was put through a spin column and diluted with buffer A to ~0.3 μM. The ribosome initiation complexes were made by mixing 2 μM 70S ribosome (mutant or wild type), 4 μM IF1, 2 μM IF2, 4 μM IF3, 4 μM fMet-$tRNA^{fMet}$, 6 μM m291, and 2 μM GTP in buffer A and incubating at 37°C for 30 min. About 17 μL of the EF-Tu ternary complex was mixed with an equal volume of the initiation complex and then the reaction was quenched with 40% formic acid after varying time intervals. GDP is separated from GTP by running a TLC plate with the different samples[7].

RESULTS & DISCUSSION:

First it was determined that none of the 8 mutations were dominant lethal in *E.coli* cells.. In order to investigate the interactions between elongation factors and the ribosome, I determined how the mutations, A55C, G357U, and U367G, on h5 affect translocation (EF-G function) and decoding (EF-Tu function). The three mutations were chosen to use for the experiments because they were the mutations that abolished translation to the greatest extent. The results gave insight as to

whether or not the two factors have differentiated in the way that they interact with the ribosome. And they also helped distinguish the reason why these mutations virtually abolish translation *in vivo*.

The Effect of h5 Mutations on EF-G Catalyzed Translocation

To test the effect of the h5 mutations on EF-G catalyzed translocation, the rate of tRNA-mRNA movement under a single-turnover condition was measured. 3'pyrene-labeled mRNA was used, which allowed the movement to be monitored by fluorescence stopped-flow. Control or mutant ribosomes with the pyrene-labeled mRNA were mixed with EF-G. A decrease in pyrene fluorescence was recorded and fit to an exponential function in order to reveal the reaction rate of translocation in each ribosome (Figure 2A). In this assay, the observed decrease in fluorescence showed biphastic kinetics with similar amplitudes for the fast and slow phases. Since it has been shown that no fluorescence change is seen in the presence of viomycin[6] an antibiotic known to block codon-anticodon movement without affecting EF-G binding, GTP hydrolysis or Pi release, it can be assumed that the fast phase is caused by codon-anticodon movement. The slow-phase might be caused by a conformational change in the way the fluorophore moves within the ribosome. Since the rate is biphasic, it was necessary to fit the curve to a double exponential function as opposed to a single exponential function. The residuals of both the single exponential fitting and the double exponential fitting show that the double exponential is indeed the better fit (figures 2B & 2C).

When comparing the apparent rates of EF-G catalyzed translocation in the presence of 1 μM EF-G, mutations A55C and U367G appeared to have a minimal effect on the rate (Figure 3). However, it appears that mutation G357U does affect the rate modestly, which may reflect a defect in EF-G binding. To investigate this further, the apparent rates of translocation were determined at various EF-G concentrations (from 1 μM to 6 μM) and these values were then fit to an equation ($k_{app} = (k_{cat}\bullet[\text{EF-G}])/(K_M + [\text{EF-G}])$)[6] in order to determine the k_{cat} and K_M parameters (figure 3). While U367G had little effect on these kinetic parameters, G357U appeared to increase the K_M substantially. However, it is worth noting that the stopped-flow machine was

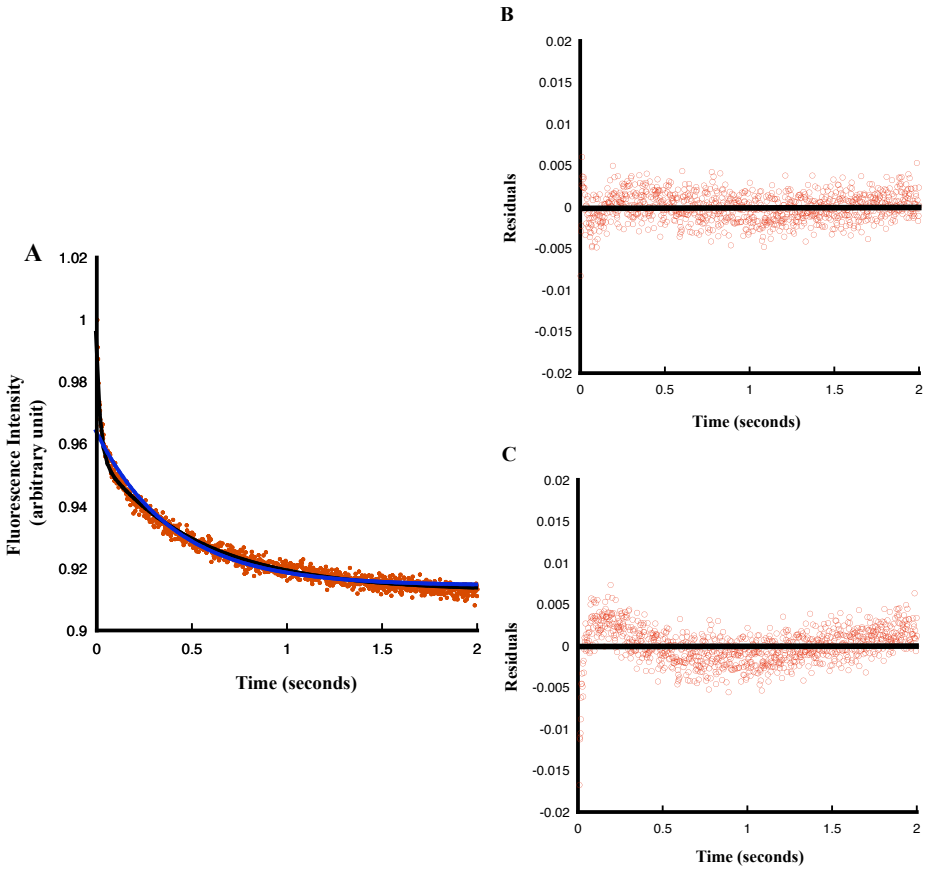

Figure 2. Effect of h5 mutations on EF-G catalyzed translocation. (A) Example of wild type ribosome translocation graph. Data obtained under 6 mM EF-G concentration. Blue line is single exponential fitting and black line is double exponential fitting. (B) Residues of the double exponential fitting plotted against time. (C) Residuals of the single exponential fitting plotted against time.

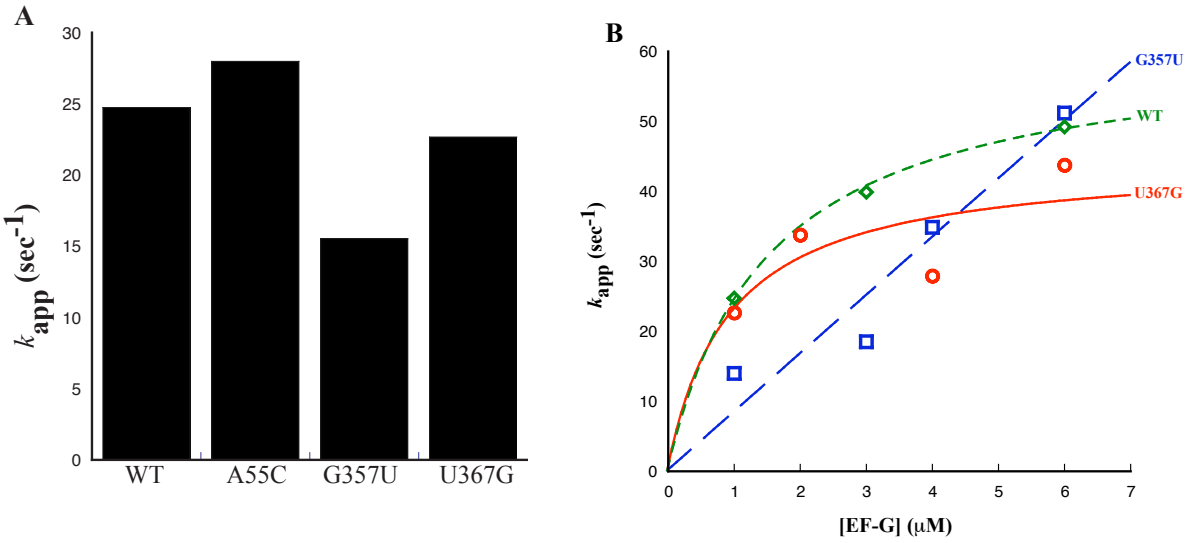

Figure 3. Effect of h5 mutations on EF-G catalyzed translocation. (A) EF-G catalyzed translocation rates at 1 μM EF-G concentration. The mutants show a minimal affect on the apparent rate with the exception of U357G. (B) Plot of k_{app} versus EF-G concentration for WT (green), A55C (red) and G357U (blue) ribosomes.

beginning to show signs of inaccuracy for later measurements (mutant ribosomes at EF-G concentrations $2 \leq x \geq 5$). Therefore, the large K_M seen for G357U might be a technological flaw; so more testing is required after the machine has been repaired. Also mutant A55C had highly variable k_{app} values, so it was not plotted on a graph. In order to obtain complete kinetic parameters on the mutant and wild type ribosomes, more tests need to be run with the stopped-flow device at varying concentrations of EF-G.

The Effect of h5 Mutations on EF-Tu Catalyzed GTP hydrolysis

In order to determine the effects of the mutations on decoding, the rate of EF-Tu catalyzed GTP hydrolysis was measured using rapid-quench. GDP production was measured at each time point by running the samples on TLC plates and determining the percentage of GDP present (Figure 4). The percentage of GDP formed was plotted against reaction time and fitted to an exponential function, from which the apparent rate of GTP hydrolysis was calculated for the mutant and wild type ribosomes (Figure 5). The calculated apparent rate for GTP hydrolysis with the wild type ribosome is 0.5 ± 0.1 sec^{-1}, while the apparent rate for mutants A55C and U367G are 0.05 ± 0.01 sec^{-1} and 0.06 ± 0.01 sec^{-1} respectively (mean SEM). These data provide evidence that nucleotides A55 and U367 are both critical for EF-Tu-dependent decoding.

CONCLUSIONS:

Based on the results obtained in the research, it can be concluded that defects in EF-Tu-dependent decoding are most likely the cause of the loss of translational activity observed when positions 55, 357 and 367 are mutated. The large effects on decoding may stem from reduced binding affinity of ternary complex (EF-Tu.GTP.aa-tRNA). It can also be concluded that EF-G-dependent translocation is minimally affected by the mutations in h5; however, there is some evidence to suggest that G357U may increase the K_M of the reaction. In general, the findings give strong indication that EF-G and EF-Tu have evolved to interact with h5 through different mechanisms and interactions. This knowledge provides direct functional evidence about the degree to which these three residues are important in decoding and translocation. This information will add to our

growing knowledge of translation at the molecular level and may lead to useful tools for protein engineering.

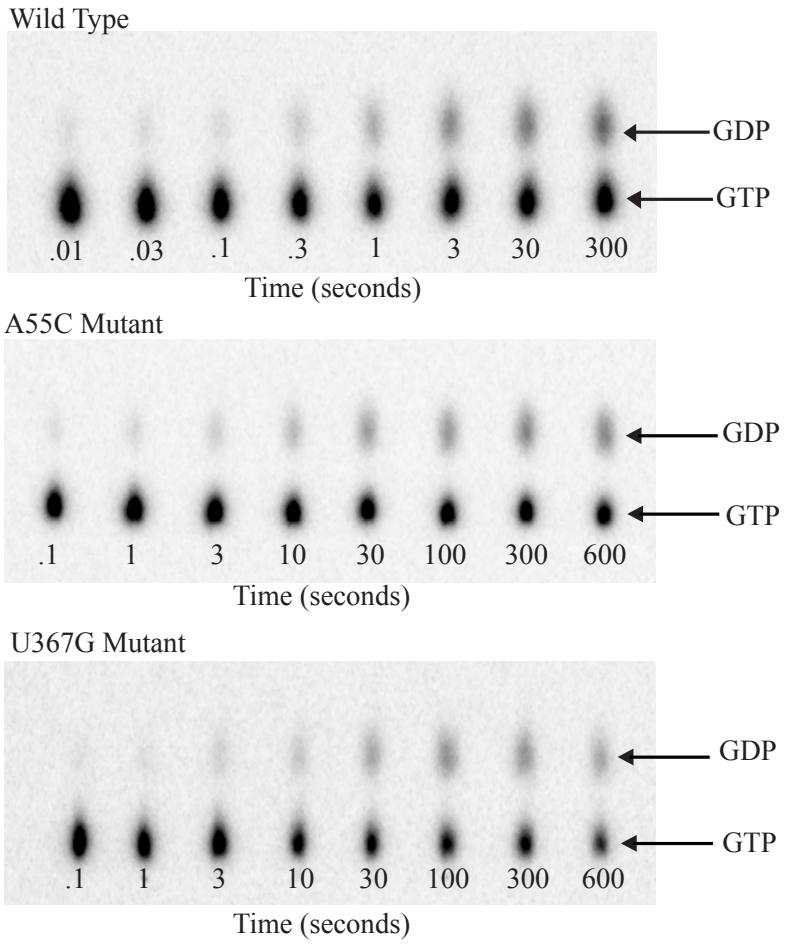

Figure 4. Effects of h5 mutations on EF-Tu catalyzed GTP hydrolysis. α-[^{32}P]-GTP was used to measure the rate of GTP-hydrolysis at different time points in a single-turnover condition. As shown, the percentage of GDP increases over a given amount of time and eventually levels off.

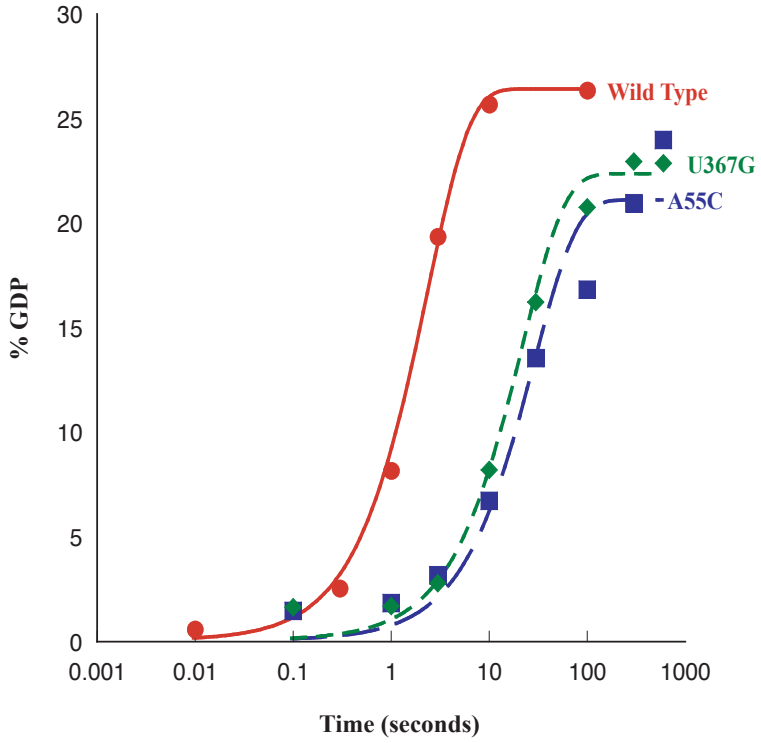

Figure 5. The effect of h5 mutations on EF-Tu catalyzed GTP hydrolysis. This graph plots the % of GDP measured on the TLC plates against time. As shown, the wild type ribosome appears to finish GTP hydrolysis much faster than the two mutants. This may reflect a defect in EF-Tu binding.

References:

1. Gao, Y., Selmer, M., Dunham, C. M., Weixlbaumer, A., Kelley, A., & Ramakrishnan, V. (2009). The structure of the ribosome with elongation factor G trapped in the posttranslocational state. *Science*, *326*, 694-699.
2. Ledoux, S., & Uhlenbeck, O. C. (2008). [3'-^{32}P]-labeling tRNA with nucleotidyltransferase for assaying aminoacylation and peptide bond formation.*Science Direct*, 74-80.
3. Mcclory, S. P., Leisring, J. M., Qin, D., & Fredrick, K. (2010). Missense suppressor mutations in 16S rRNA reveal the importance of helices h8 and h14 in aminoacyl-tRNA selection. *RNA*, 1925-1934.
4. Schmeing, M., & Ramakrishnan, V. (2009). What recent ribosome structures have revealed about the mechanism of translation. *Nature*, *461*, 1234-1239.
5. Schmeing, M., Voorhees, R., Kelley, A. C., Gao, Y., Murphy, F. V., Weir, J. R., & Ramakrishnan, V. (2009). The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science*, *326*, 688-693.
6. Studer, S. M., Feinberg, J. S., & Joseph, S. (2003). Rapid Kinetic Analysis of EF-G-dependent mRNA Translocation in the Ribosome. *J. Molecular Biology*, *327*, 369-381.
7. Vorstenbosch, Erik, Tillmann Pape, Marina Rodnina, Barend Kraal, and Wolfgang Wintermeyer. "The G222D Mutation in Elongation Factor Tu Inhibits the Codon-induced Conformational Changes Leading to GTPase Activation on the Ribosome." *The EMBO Journal* 15.23 (1996): 6766-774.
8. Walker, S. E., Shoji, S., Pan, D., Cooperman, B. S., & Fredrick, K. (2008). Role of hybrid tRNA-binding states in ribosomal translocation. *PNAS*, *105*, 9192-9197.
9. Youngman, E. M., & Green, R. (2005). Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis. *Methods*, *36*, 305-312.